BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

06-25-04

1638

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER OF PRIORITY DOCUMENT		Docket Number: 13101/48801	Confirmation No.: 4447
Application Number 09/978,273	Filing Date October 15, 2001	Examiner Cynthia E. Collins	Art Unit 1638
Invention Title **PLANT CELL DEATH SYSTEM**		Inventor(s) Christopher John Robert THOMAS et al.	

By EXPRESS MAIL - Label No. EV 321890380 US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

S I R:

A claim of priority pursuant to 35 U.S.C. § 119 of Application No. GB 0025225.4 filed on October 14, 2000 in Great Britain was previously made. To complete the claim of priority, a certified copy of the priority application is enclosed.

Accompanying this paper is a copy of (1) a Power of Attorney by Assignee of Entire Interest (Revocation of Prior Powers and Appointment of New Power) and (2) a Certificate under 37 C.F.R. 3.73(b) designating the undersigned attorneys as the Attorneys of Record in this case, which were filed contemporaneously herewith.

If any fees are necessary, the Commissioner is hereby authorized to charge Deposit Account No. 11–0600 of Kenyon & Kenyon.

Dated: June 23, 2004

By: Anthony Giaccio, Reg. No. 39,684

KENYON & KENYON
One Broadway
New York, N.Y. 10004
(212) 425-7200 (telephone)
(212) 425-5288 (facsimile)
Customer No. 26646

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed D. Mahoney

Dated 29 August 2003

Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
F
14 SEP 2000
NEWPORT

The Patent Office

1/77

160CT00 E576081-1 D01886
P01/7700 0.00-0025225.4

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — RD-ATC-28

2. Patent application number *(The Patent Office will fill in this part)* — **0025225.4** — 14 OCT 2000

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Advanced Technologies (Cambridge) Limited
Globe House
1 Water Street
London WC2R 3LA
England

Patents ADP number *(if you know it)* 7577356002

If the applicant is a corporate body, give the country/state of its incorporation — England & Wales

4. Title of the invention

PLANT CELL DEATH SYSTEM

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Margot Ruth WALFORD
Patents Department
British American Tobacco (Investments) Limited
R&D Centre
Regents Park Road
Southampton SO15 8TL
England

Patents ADP number *(if you know it)* 5819198004

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	41 pages x 2
Claim(s)	(1 page (Table 1) x 2 (p54) / 12 pages (sequences) x 2 (p55-66)) other docs
Abstract	–
Drawing(s)	12 sheets x 2

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature MRWalford Date 13.10.00

Margot Ruth WALFORD – Asistant Company Secretary

12. Name and daytime telephone number of person to contact in the United Kingdom: Mrs. Margot Walford – 023 80793730

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

Plant Cell Death System

The present invention relates to a plant cell death system, and in particular to transgenic plants which harbour within their genome a chimaeric gene which when expressed produces a cytotoxic protein.

One of the means open to plant breeders attempting to produce new cultivars is the production of hybrids between existing cultivars containing desirable traits. Hybrids are generally superior in a variety of characteristics to either parent, a phenomenon known as hybrid vigour. Such hybrid crosses may be performed by manual cross pollination, a tedious and time consuming procedure.

During the production of such hybrid crosses the prevention of self pollination is vital. To achieve this, the female parent may be emasculated by hand, e.g. in the production of hybrid corn by de-tasseling. However, the large scale emasculation of species with hermaphrodite flowers is economically unfeasible. Female parent lines (male sterile) may also be generated by genetic male sterility, a known trait in many plants, usually being recessive and monogenic. The problem with this approach is that it is difficult to obtain pure lines of male sterile parents for every cross. The most widely used system of producing male sterility for use in hybrid production is cytoplasmic male sterility (cms). In

this case cytoplasmic factors are responsible for pollen abortion. In crops where cms has been identified in the germplasm it has been used extensively e.g. maize, sunflower. There are several disadvantages of the system: male sterile cytoplasm may be associated with other undesirable characteristics e.g. T-cytoplasm in maize and susceptibility to *Helminthosporium maydis*; its application requires isogenic maintainer male fertile lines to propagate the male parent; and it is limited to species in which a cytoplasmic source of sterility is available.

Another advantage of a male sterility system would be the production of pollen-free plants. This would be desirable in a number of ornamental flower varieties, and would also have application in the containment of genetic traits by the prevention of outcrossing.

A further desirable property of a sterility system is that female sterile plants could be produced such that fruit development would occur in the absence of seed set. Seedless fruit varieties would be advantageous for processing, e.g. tomatoes, and also desirable to the consumer, e.g. melon. Seedless varieties are available and there are established breeding programmes, but the development of seedless fruit has been limited by the availability of the appropriate germplasm in many species.

In cases where a genetic source of sterility is not available or is otherwise unfeasible, a genetic modification approach could provide sterility by providing a cell death system whereby necrosis occurs in specific cells in the reproductive tissues.

WO 89/10396 discloses a plant cell death system wherein a chimaeric gene is introduced into a plant, which chimaeric gene comprises an anther specific promoter attached to a RNAse protein or polypeptide which, when expressed, causes disruption of cell metabolism. Thus, expression of the chimaeric gene results in necrosis of the anther cells and results in male sterility in the plants.

The present cell death system could be used to provide female sterility in plants, whereby the target site may be the ovule of the plant.

WO 93/18170 and WO 92/04453 disclose plant cell death systems which are specific to controlling nematode infection. In WO 93/18170 and WO 92/04453 a gene comprising a coding sequence, which coding sequence encodes for a product which is disruptive of nematode attack is introduced into a host plant species. The gene further comprises a promoter region, which promoter region controls the expression of the coding sequence such that expression occurs upon nematode attack and substantially specifically within or adjacent to the nematode feeding site cells. In order to disrupt nematode attack, the

product may be either inimical to the plant cells which differentiate into nematode feeding site cells or cells adjacent thereto, or inimical to the nematodes directly.

Economically important plant parasitic nematodes include cyst nematodes, such as potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*), soybean cyst nematode (*Heterodera glycines*), beet cyst nematode (*Heterodera schachtii*) and cereal cyst nematode (*Heterodera avenae*), and root knot nematodes, such as *Meloidogyne* spp. Such plant parasitic nematodes are major pathogens of many crops world-wide, for example vegetables, food legumes, tomato, water melon, grape, peanut, tobacco and cotton.

Chemical control, cultural practices and the use of resistant plant varieties are the chief approaches to nematode control which are currently available and they are often used in an integrated manner against plant parasitic nematodes. There is a requirement for improvement in nematode control because these current approaches offer inadequate crop protection. Nematicides are of questionable environmental status and they are not always efficacious. Cultural control imposes hidden losses on growers in several ways. The wide host range of root knot nematodes limits the availability of economically satisfactory non-host crops. Effective resistant cultivars are frequently unavailable and those that the grower can use are sometimes out-performed by susceptible cultivars

at low nematode densities. Also resistance may be lost in the high soil temperatures that occur in tropical and sub-tropical environments.

Other applications of plant cell death systems can be envisioned. For example the target site may be specific parts of the flower, thereby altering the morphology of the flower. Alternatively the target site may be lateral roots, thorns or stinging hairs. Abscission of leaf or fruit might be achieved by the targeting the abscission zone of the leaf or the fruit. Facilitating the release of seeds from plants, by targeting the funicle might be achievable. By targeting other organs such as trichomes, which trichomes are typically glandular, the production of chemical substances by the trichomes can be cessated or prevented. Another application might be the inducible abscission of roots, leaves, flowers, or fruit at the end of the growing season.

Ribosome-inactivating proteins (RIPs) are a group of toxic plant proteins that catalytically inactivate eukaryotic ribosomes (Stirpe and Barbieri 1986). RIPs function as *N*-glycosidases to remove a specific adenine in a conserved loop of the large rRNA, and thereby prevent binding of Elongation Factor 2, thus blocking cellular protein synthesis.

Three forms of RIPs have been described. Type 1 RIPs such as pokeweed antiviral protein and barley translation inhibitor are each comprised of a single polypeptide chain, each with an

approximate M_r value of 30,000. Type 2 RIPs such as ricin, abrin and modeccin each comprise two polypeptide chains; one polypeptide with RIP activity (A-chain) is linked by a disulphide bond to a galactose-binding lectin (B-chain; Stirpe *et al* 1978). The M_r value of each Type 2 RIP is approximately 60,000.

Maize RIP, which is found in the endosperm of maize (*Zea mays*) seeds, is a Type 3 RIP. This RIP is synthesised as a single polypeptide chain, but subsequently undergoes proteolytic cleavage to release two active peptide domains. Maize RIP comprises two domains, the α domain and the β domain, which domains are separated in the inactive form of maize-RIP (i.e. maize pro-RIP) by a central peptide spacer and are flanked by N and C terminal peptides. The α domain is located towards the N terminus of the pro-RIP; the β domain is located towards the C terminus. During seed germination, the maize pro-RIP is activated by proteolytic cleavage of the N and C terminal peptides together with the central peptide spacer such that the two domains form the mature (active) maize RIP (US 5,248,606). Cleavage is effected by endogenous proteases.

The present invention provides a method of producing a transgenic plant which harbours within the genome of the plant a chimaeric gene, the expression of which gene causes plant cytotoxicity, wherein a plant is transformed with a chimaeric

gene comprising a promoter, which promoter is induced at and/or adjacent to a target site, operably linked to a coding sequence, which coding sequence encodes a maize ribosome inactivating protein or a part thereof.

As used herein "part" means a part of the gene coding for maize RIP, which part is active in inhibiting protein synthesis .

The present invention further provides a plant transformed with a chimaeric gene comprising a promoter, which promoter is induced at and/or adjacent to a target site, operably linked to a coding sequence, which coding sequence encodes a maize ribosome inactivating protein or a part thereof.

The present invention yet further provides a plant cell transformed with a chimaeric gene comprising a promoter, which promoter is induced at and/or adjacent to a target site, operably linked to a coding sequence, which coding sequence encodes a maize ribosome inactivating protein or a part thereof.

The present invention also provides a DNA isolate of a chimaeric gene comprising a promoter, which promoter is induced at and/or adjacent to a target site, operably linked to a coding sequence, which coding sequence encodes a maize ribosome inactivating protein or a part thereof.

The present invention further provides a biologically functional expression vehicle containing a chimaeric gene comprising a promoter, which promoter is induced at and/or adjacent to a target site, operably linked to a coding sequence, which coding sequence encodes a maize ribosome inactivating protein or a part thereof.

The coding sequence of the maize ribosome inactivating protein disclosed in this invention may comprise the entire pro-RIP sequence as described above comprising the N-terminal peptide, the α domain, the central spacer peptide, the β domain, and the C-terminal peptide.

The coding sequence of the maize ribosome inactivating protein may alternatively comprise a recombinant "mature" RIP, described herein as "RIP-P", comprising the α domain and the β domain arranged contiguously, i.e. the N- and C- terminal extensions and the central spacer peptide are removed. The present invention demonstrates that the provision of a functionally active RIP molecule is not dependent upon any conformational constraints placed upon it by the intact pro-RIP molecule or by the cleavage reaction. Functional activity in cell-free translation systems of recombinant RIP molecules lacking the central spacer and terminal peptides has been described in US Patent Nos. 5248606 and 5646026.

The coding sequence of the maize ribosome inactivating protein may preferably otherwise comprise a recombinant RIP

comprising the α domain only, or a recombinant RIP comprising the β domain only, the α domain or domain alone being a part of the maize ribosome activating protein having the required functionality. The α domain of the maize ribosome inactivating protein is that domain which is situated towards the N terminal end of the maize ribosome inactivating protein, whilst the β domain of the maize ribosome inactivating protein is that domain which is situated towards the C terminal end of the maize ribosome inactivating protein.

Without wishing to be bound by theory, the α domain is thought to comprise ribosome recognition and binding regions, whereas the β domain is thought to comprise the catalytic site necessary for depurination/cleavage of the ribosome. Prior to the present invention it was thought that the β domain was essential to provide an active maize ribosome inactivating protein. Thus, it was surprising to find that the inclusion of the α domain alone (i.e. without the β domain) could be used to disrupt plant ribosome function and result in plant cell necrosis.

The nucleotide sequence of the pro-RIP coding sequence is that identified in SEQ ID NO:1 or a coding sequence which is homologous thereto ; the nucleotide sequence of the RIP-P coding sequence is that identified in SEQ ID NO:2 or a coding sequence which is homologous thereto; the nucleotide sequence

of the α domain coding sequence is that identified in SEQ ID NO:3 or a coding sequence which is homologous thereto; the nucleotide sequence of the β domain coding sequence is that identified in SEQ ID NO:4 or a coding sequence which is homologous thereto.

Depending on the homology of the nucleotide sequences required, different conditions of stringencies may be used in the hybridisation procedure used to screen for similar sequences. By way of example and not limitation, hybridisation procedures using conditions of high stringency are as follows: hybridisation to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. *et al*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley and Sons, Inc., New York, at p. 2.10.3). Other conditions of high stringency which may be used are well known in the art. Hybridisation procedures using conditions of moderate stringency that may be used are as follows: hybridisation to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel *et al*, 1989, *supra*). Other conditions of moderate stringency which may be used are well-known in the art. Other solutions such as Standard Saline Citrate (SSC) or (Saline Sodium Phosphate EDTA) (SSPE) can be used in the hybridisation procedures.

Suitable homologous sequences are sequences that are at least 60%, preferably 70% and more preferably 80%, or more homologous with each sequence listed herein, under moderate stringency conditions.

Suitably, the chimaeric gene further comprises a 3' untranslated, terminator sequence. The terminator sequence may be obtained from plant, bacterial or viral genes. Suitable terminator sequences are the pea *rbcS* E9 terminator sequence, the *nos* terminator sequence derived from the nopaline synthase gene of *Agrobacterium tumefaciens* and the 35S terminator sequence from cauliflower mosaic virus, for example. A person skilled in the art will be readily aware of other suitable terminator sequences. In addition, the chimaeric gene may optionally comprise transcriptional or translational enhancer sequences,such as those described in International Patent Application, Publication No. WO 97/20056, intracellular targeting sequences and introns, for example, as well as nucleotide sequences operable to facilitate the transformation process and the stable expression of the chimaeric gene, such as T-DNA border regions, matrix attachment regions and excision/recombination sequences.

The present invention also provides a two-component system to achieve cell death. Such two-component systems are described in International Patent Applications WO 98/32325 and WO 93/18170 whereby two or more transgenes are employed such

that the combined effects of the their expression products lead to cell death. The individual components are inactive or harmless in isolation, but exhibit a cytotoxic effect when present together. Each transgene is driven by a separate promoter, the promoters being selected such that their expression profiles overlap at the desired target site of cell death, but not in other tissues. The present invention describes a two component system comprising separately the α domain and the β domain of maize ribosomal inactivating protein, each as a separate transgene construct driven by a separate promoter. Examples of suitable promoters are described below and others will be known to those skilled in the art. Such a two-component system may be produced by the crossing of two plants each containing one component, by the stacking of transgenes through sequential or simultaneous transformation with two transgene constructs, or by transformation with a construct containing both components in a single cassette.

Preferably, the promoter is induced specifically or substantially specifically at and/or adjacent to the target site. If the promoter is induced other than at the target site and/or the cells adjacent to the target site, the promoter is preferably predominantly expressed at the target site and/or adjacent thereto.

In accordance with a first embodiment of the present invention, the target site may be a nematode feeding site. When it is the case that the target site is a nematode feeding site, the promoter selected is one which is induced at and/or adjacent to the nematode feeding site. Such a promoter is preferably induced upon nematode infection of the plant. An example of a suitable promoter is the KNT1 promoter. The isolation of the KNT1 promoter is described in NZ Patent No. 260511 and the method is further recited below. Other suitable promoters include the TobRB7 promoter and the Lemmi promoters. The isolation of the TobRB7 promoter is taught in International Patent Application WO 94/17194, and the isolation of the Lemmi promoters are disclosed in International Patent Application WO 92/21757.

The nematode feeding site may be comprised of, for example, plant cells at the local site of infection which later redifferentiate to form a syncytium (in the case of cyst nematodes) or the giant cells and/or the accompanying hypertrophic cells (in the case of root knot nematodes), and/or one or more of the syncytium cells, the giant cells and the accompanying hypertrophic cells.

By targeting the nematode feeding site a nematode resistant plant may be obtained. By the term "nematode resistant plant" it is meant a plant which upon infection by plant parasitic nematodes is capable of preventing, slowing or

otherwise adversely affecting the growth and development of nematodes that attack the plant, thereby preventing economically significant densities of plant parasitic nematodes from building up during a single crop growing period. That is to say that the nematodes may for, example die or the nematodes' life cycle may be slowed resulting in a delay in the time taken to reach maturity and hence produce eggs, or the mature female nematodes may be of reduced size and thus have a lower egg-laying capacity as egg laying only commences after female nematodes have reached a critical, minimum size.

The present invention is applicable to, but in no way limited to, use with the following nematode species: *Globodera* spp., *Heterodera* spp. and *Meloidogyne spp*.

In accordance with a second aspect of the present invention, instead of nematode resistance the method is directed to effecting male sterility in plants. For example, the target site may be one or more of a plant's pollen, anther or tapetum. When it is the case that the target site is tapetum for example, the promoter selected is one that is induced in and/or adjacent totapetum. An example of a suitable tapetum promoter is the tobacco TA29 promoter as disclosed in Mariani et al (1990). Anther specific promoters are disclosed in Twell et al (1991).

In accordance with a third aspect of the present invention, the method is directed to effecting female sterility in plants. For example, the target site may be the ovule of the plant. That is to say, the promoter selected is one that is induced in and/or adjacent to the ovule. An example of a suitable promoter is the AGL15 promoter as disclosed in Perry et al, 1996.

According to a fourth aspect of the present invention, the morphology of the flower of a plant is manipulated. For example, the target site may be specific parts of the flower, the aim being that when these specific parts of the flower do not develop the morphology of the flower is changed. In that instance, the promoter selected is one that is induced in and/or adjacent to the sepal, carpel, petal, and/or stamen. Examples of suitable promotersare the found in the agamous, apetala3, globosa, pistillata and deficiens genes (Sieburth and Meyerowitz, 1997; Samach et al, 1997, and references therein).

In accordance with a fifth aspect of the present invention, the method is used to assist in or promote leaf and/or fruit abscission in plants. For example, the target site may be the abscission zone of the leaf and/or the fruit. Thus, the promoter selected is one that is induced in and/or adjacent to such an abscission zone.

A sixth aspect of the present invention is the targeting of trichomes, which trichomes are typically glandular. The promoter selected is one that is induced in and/or adjacent to the trichomes. By causing necrosis of the trichomes of the plant the production of chemical substances by the trichome can be cessated or prevented. A seventh aspect of the present invention is the targeting of lateral roots, thorns or stinging hairs.

In accordance with an eighth aspect of the present invention the method is directed to the control of virus infections. During virus infections there are a number of genes which are induced specifically, or substantially specifically, within the cells actually infected by the virus. The promoter selected is one that is induced in and/or adjacent to the cells infected by the virus

According to a ninth aspect of the present invention the method is directed to facilitating the release of seeds from plants, by targeting the seeds. During seed development there are a number of genes which are induced specifically, or substantially specifically, within certain cells/parts of the seed.

In accordance with a tenth aspect of the present invention a promoter that is externally inducible and that is induced in the, for example, roots of the plant is selected. Such a promoter could be used to effect root abscission at the

end of a growing season. Comparable promoters induced in, for example, leaf petioles, pedicels or peduncles, could be used to effect abscission of leaves, flowers, or fruit at the end of the growing season.

Techniques for transforming plants are well known within the art and include *Agrobacterium*-mediated transformation, for example. Typically, in *Agrobacterium*-mediated transformation a binary vector carrying a foreign DNA of interest, i.e. a chimaeric gene, is transferred from an appropriate *Agrobacterium* strain to a target plant by the co-cultivation of the *Agrobacterium* with explants from the target plant. Transformed plant tissue is then regenerated on selection media, which selection media comprises a selectable marker and plant growth hormones.

Further suitable transformation methods include direct gene transfer into protoplasts using polyethylene glycol or electroporation techniques, particle bombardment, micro-injection and the use of silicon carbide fibres for example.

Suitable plant species which may be transformed in accordance with the present invention include, but are not limited to, rice, wheat, maize, potato, tobacco, sugar beet, soybean, canola, tomato, peanut, cotton, vine, watermelon, papaya, vegetables and food legumes.

EXAMPLES

Cloning and sequencing of the maize ribosome inactivating protein

Genomic DNA was extracted from 14 day-old seedlings of maize variety Earli King.

Primers were designed from the nucleotide sequence of maize RIP genomic DNA to generate variants of RIP sequences, by eliminating the N- and C-terminal regions and central spacer. The primers were also designed to remove a *Sac*I restriction site in the RIP sequence and so facilitate cloning (Figure 12). RIP sequences were amplified with *Pfu* polymerase.

The Pro RIP sequence (SEQ. ID. No. 1) was obtained by PCR of the genomic DNA as follows:

Two PCR reactions were performed using primers PRORIPBF (SEQ. ID. No. 5) plus RIPSDR (SEQ. ID. No.14), and RIPSDF (SEQ. ID. No. 13) plus PRORIPSR (SEQ. ID. No. 6) respectively. The PCR products were gel purified and combined. Overlap extension followed by PCR amplification using primers PRORIPBF (SEQ. ID. No. 5) and PRORIPSR (SEQ. ID. No. 6) resulted in the full length ProRIP sequence.

PCR of ProRIP with primers RIP1BF (SEQ. ID. No. 7) and RIP2SR (SEQ. ID. No. 8) resulted in a PCR product of approximately 800bp, corresponding to the RIPα, central

spacer, and RIPβ domains. This product ("RIP-CD") was digested with restriction endonucleases *Xba*I and *Sal*I, gel purified, ligated into the vector pBluescript, transformed into *E.coli* XL1-Blue cells, and sequenced. The sequence was identical to that of the equivalent region of the maize RIP DNA.

The central spacer region was removed as follows: The RIP-CD DNA was amplified in two PCR reactions using the primers RIP1BF (SEQ. ID. No. 7) plus RIPCDR (SEQ. ID. No. 12) and RIPCDF (SEQ. ID. No. 11) plus RIP2SR (SEQ. ID. No. 8). The PCR products were gel purified and combined. Overlap extension followed by PCR with primers RIP1BF (SEQ. ID. No. 7) and RIP2SR (SEQ. ID. No. 8) resulted in the fully processed RIP (RIP-P). RIP-P was digested with restriction endonucleases *Xba*I and *Sal*I, gel purified, ligated into the vector pBluescript, transformed into *E.coli* XL1-Blue cells, and sequenced. The RIP-P sequence is identified herein as SEQ. ID. No. 2).

Further PCR reactions were carried out on RIP-CD using either primers RIP1BF (SEQ. ID. No. 7) plus RIP1SR (SEQ. ID. No. 9) or primers RIP2BF (SEQ. ID. No. 10) plus RIP2SR (SEQ. ID. No.8), to amplify the RIPα or RIPβ domains respectively. RIPα or RIPβ was digested with restriction endonucleases *Xba*I and *Sal*I, gel purified, ligated into the vector pBluescript,

transformed into *E.coli* XL1-Blue cells, and sequenced. The sequences of RIPα and RIPβ are identified as SEQ. ID. No. 3 and ID. No. 4 respectively.

Production of constructs comprising the maize ribosome inactivating protein for assessment in transient protoplast assays

Constructs containing the cauliflower mosaic virus 35S constitutive promoter linked to a coding sequence derived from the maize ribosome inactivating protein and a nos terminator sequence were produced in a pDE4 derived vector (Denecke *et al*, 1990) (see Figure 11) suitable for use in a transient expression protoplast system. The following constructs were made and schematics are shown in Figure 5:

1. pDE4 35S/ Pro RIP

3. pDE4 35S/RIP P ("mature" RIP)

4. pDE4 35S/RIP α

5. pDE4 35S/RIP β

Transient assay of RIP constructs in tobacco protoplasts

In this assay ribosome inactivating protein-mediated ribosome inactivation was detected by way of assessing GUS

protein synthesis. Protoplasts were prepared from leaves of *in vitro*-maintained tobacco plants and were electroporated with the RIP constructs. The protein translation efficiency of ribosomes in the tobacco protoplasts was evaluated with a GUS reporter gene in a pDE4 construct under the control of a CaMV 35S promoter. A GUS pDE4 construct was co-electroporated with each RIP construct. In order to obtain values for the optimum levels of GUS protein synthesis in protoplasts in competition with a second protein, a GUS-positive control was used, wherein a non-toxic BiP protein construct, pDE800 (Leborgna-Castel *et al*, 1999) was co-electroporated into tobacco protoplasts together with the GUS pDE4 construct. A GUS-negative control was also used, wherein an empty pDE4 vector was electroporated into tobacco protoplasts together the non-toxic BiP chaperone protein construct.

(i) Mature maize RIP-P.

Maize RIP-P constructs were co-electroporated with the GUS construct into tobacco protoplasts. The effect of maize RIP activity on ribosomes was assayed by measuring the levels of GUS activity after 24 hours of expression (Figure 1). The dosage effect of the construct was assayed by electroporating different quantities (0.02µg to 20µg) of the maize RIP construct (Figure 2).

The results demonstrate that mature maize RIP protein inactivates tobacco ribosomes efficiently. Consequently only basal levels of GUS activity are observed in comparison with the GUS positive control.

The efficiency of ribosome inactivation depended on the amount of input DNA electroporated into the protoplasts. Higher amounts of RIP DNA (20μg) induced ribosome inactivation rapidly and only basal levels of GUS activity was observed. Lower amounts of input DNA (0.02μg) resulted in much greater residual GUS activity, possibly due to the longer time taken for the RIP to reach critical protein concentrations and completely inactivate the ribosomes.

(ii) Maize RIP activity on different ribosomes of the cell:

An experiment was conducted to test whether the maize RIP-P depurinates both the ribosomes on the Endoplasmic Reticulum (ER) and those in cytosol. As an example of a protein synthesised on the ER, α-Amylase protein possesses an N-terminal signal sequence and the protein is secreted via the ER. An α-Amylase construct was co-electroporated into protoplasts along with the RIP-P construct, and a GUS construct was also used for comparison. After 24 hours expression, an amylase assay was performed (Figure 3).

The results indicated no significant difference between the effect of RIP-P on the α-Amylase and on the GUS activities. The mature maize RIP-P inactivated all ribosomes irrespective of their cellular location. The results of these assays with maize RIP-P indicate that the enzyme is highly effective to induce complete ribosome inactivation and subsequent cell attenuation or cell death.

(iii) The separate maize RIP domains.

Maize RIPα and RIPβ polypeptide regions were also expressed either individually or in combination in tobacco protoplasts (Figure 4).

Expression of RIPα protein alone surprisingly resulted in a significant reduction in the GUS activity. In contrast, expression of RIPβ protein alone was found to be inactive. On the basis of structural predictions, RIPα contains the RNA recognition motif and ribosome binding domain regions, but not the critical catalytic residue site. RIPα protein may be preventing protein translation by binding to ribosomes and preventing the protein translation. This would imply that RIPα is adopting a correctly folded conformation and is capable of specific molecular recognition. RIPβ contains the active site residue necessary for ribosome depurination and

might not be expected to be capable of interacting with the ribosome.

Expression of both RIPα and RIPβ simultaneously in protoplasts resulted in further reduction of GUS activities in comparison with RIPα and RIPβ proteins alone. This implies that the two peptides are able to interact and facilitate ribosome depurination.

Isolation of a promoter that is induced at and/or adjacent to a target site

A method is hereby presented for the isolation of a promoter, which method is by way of an example. Alternative methods for the isolation of a suitable promoter for use in the present invention will be readily available to the skilled person, some of which methods are referenced above.

A method for the isolation of the KNT1 promoter is as follows:

Growth and Infection of Tobacco Plants

Seed of C319 tobacco were germinated on Fisons F1 compost under conditions as follows: light intensity of 4500 to 5000 lux; 16hr day/8hr night; temperature 20-25°C. After *c.* 3 weeks seedlings were gently washed in tap water to remove soil and transferred to pouches (Northrup-King), 2 plants per pouch, and grown for a further week in a Conviron at 25°C with lighting as above. Roots were lifted from the back of the pouch and supported with Whatman GF/A glassfibre paper at

their tips. Three-day-old nematodes (*M.javanica*) were then delivered to the tips of these roots in 10µl (50 nematodes) aliquots and a second piece of GF/A paper was placed on top to fully encapsulate the root tip. Following 24 hours post infection, the GF/A paper was removed to ensure synchronous infection. Following 3 days post infection the root knots were dissected out (leaving healthy root and root tip tissue behind) and frozen immediately in liquid nitrogen. Approximately 0.5 -1.0g of infected root tissue was harvested from 80 inoculated plants.

Staining for visualisation of nematodes in infected roots

The quality of infection was established by determining the number of nematodes infecting per root tip. Roots were harvested from 3 day post infected plants and immersed for 90 seconds in lactophenol containing 0.1% Cotton Blue at 95°C. Following a 5 second rinse in water, the roots were placed in lactophenol at room temperature (RT) for 3-4 days to clear. Stained nematodes were visualised using light microscopy.

RNA isolation from healthy and infected root tissue

Root tissue was ground to a fine powder in a liquid nitrogen chilled pestle and mortar. Approximate 100mg aliquots were then transferred to similarly chilled microfuge tubes and 300µl of hot phenol extraction buffer (50% phenol, 50% extraction buffer: 0.1M lithium chloride, 0.1M Tris-HCl pH8.0 (RT), 10mM EDTA, 1% SDS) added, and incubated at 80°C for

5 minutes. An equal volume of chloroform was then added and the homogenate microfuged for 15 minutes at 4°C. The aqueous phase was then extracted with 600µl of phenol/chloroform and microfuged as above. The aqueous phase was again removed and the RNA precipitated with an equal volume of lithium chloride at 4°C overnight. The precipitate was pelleted by microfugation for 15 minutes at RT and washed in 70% ethanol. The pellet was lyophilised, resuspended in DEPC-treated water and assayed using a spectrophotometer. RNA quality was assessed by denaturing gel electrophoresis. (Adapted from Shirzadegan et al, 1991).

Subtractive cloning of infection specific cDNAs

Poly(A)$^+$ RNA (mRNA) was isolated from 200µg total RNA samples from healthy and infected C319 root tissue using magnetic oligo dT Dynabeads according to the manufacturer's instructions. First strand cDNA synthesis was performed *in situ* on the Dynabead-bound poly(A)$^+$ fraction from healthy tissue to provide Driver DNA. First and second strand synthesis was performed *in situ* on the Dynabead-bound poly(A)$^+$ fraction from the infected tissue to provide Target DNA. All cDNA reactions were carried out using a cDNA synthesis kit according to the manufacturer's instructions (Pharmacia). Three oligonucleotides, SUB21 (5' CTCTTGCTTGAATTCGGACTA 3') (SEQ. ID. No. 15), SUB25 (5' TAGTCCGAATTCAAGCAAGAGCACA 3') (SEQ. ID. No. 16)(sequences from Duguid & Dinauer, 1990) and

LDT15 (5' GACAGAAGCGGATCCd$(T)_{15}$ 3') (SEQ. ID. No. 17)(O'Reilly, 1991) were kinased with T4 polynucleotide kinase according to Maniatis et al (1982). SUB21 and SUB25 were then annealed to form a linker which was then ligated to the Target DNA with T4 DNA ligase according to King & Blakesley (1986). Subsequently the beads carrying the Target DNA were washed extensively with TE and the second strand of the cDNA eluted at 95°C in 5xSSC.

The RNA bound to the Dynabead-bound Driver DNA was removed by heat and the eluted Target DNA hybridised to the Driver DNA at 55°C in 5xSSC for 5 hours. Non-hybridising Target DNA was separated from the bead-bound Driver DNA at room temperature (RT) as per the manufacturer's instructions, following which, hybridising Target DNA was similarly separated from the bead-bound Driver DNA at 95°C. The RT eluted Target DNA was then added back to the Driver DNA and the hybridisation repeated. This process was repeated until the amount of Target hybridising to the driver no longer exceeded the amount that did not hybridise. DNA concentrations were established using DNA Dipstick (Invitrogen) in accordance with the manufacturer's instructions.

Aliquots of the final RT-eluted fraction were used in PCR amplification (Eckert and Kunkel, 1990) to generate double-stranded cDNA for cloning into a plasmid vector. Amplification of the target DNA was achieved using primers

SUB21 (SEQ. ID. No. 15) and LDT15 (SEQ. ID. No. 17) according to the conditions described by Frohman et al, 1988. The PCR products were then ligated into *Sma*I digested pBluescript vector according to King and Blakesley (1986).

Screening of the subtractive library by Reverse Northern analysis

Recombinants were identified by colony PCR (Gussow and Clackson, 1989). The amplified inserts were Southern blotted in triplicate onto Pall Biodyne membranes and prescribed by the manufacturer. Prehybridisation and hybridisation were both carried out at 42°C in 5xSSPE, 0.05% BLOTTO, 50% formamide. Membranes were hybridised separately to cDNA probes (see below) from healthy and infected tissue and to a probe comprising amplified Target DNA from the final subtraction. Clones showing a hybridisation signal to the infected cDNA probe only, orshowing a hybridisation signal to the subtracted probe only were selected for further analysis.

cDNA probe generation

Samples of 10μg total RNA from healthy and infected tissue were treated with 2.5 units Dnase1 at 37°C for 15 minutes. The Dnase1 was then denatured at 95°C for 10 minutes before cDNA synthesis was performed using the manufacturer's protocol (Pharmacia). The RNA was then removed by the presence of 0.4M sodium hydroxide for 10 minutes at RT and the

cDNA purified through a spun Sephacryl 400HR column. Yield and concentration was determined using DNA Dipsticks (Invitrogen). The cDNA was labelled, using *c.* 35ng/probe using the standard Pharmacia oligolabelling protocol.

Northern Blotting

To determine the expression profile of the clones selected from Reverse Northerns, they were used as probes in Northern analysis of either total or poly(A)$^+$ RNA from healthy and infected roots, stems, leaves, and flowers. Total RNA blots comprised 25μg RNA per lane, whilst poly(A)$^+$ RNA blots comprised 0.5-1.0μg RNA per lane. The RNA was electrophoresed on formaldehyde gels and blotted onto Pall Biodyne B membrane as described by Fourney et al (1988). Probes were labelled and hybridised as above.

Southern blotting

To determine whether selected cDNAs were of plant or nematode origin, tobacco C319 and *M.javanica* DNA were prepared as described by Gawel and Jarret (1991). Southern blots were prepared comprising 10μg *Eco*RI and *Hin*dIII digested DNA per lane. The blots were hybridised to oligolabelled probes as described above.

***In Situ* hybridisations**

To determine the locality of expression of the cDNAs of interest at the feeding site, *in* situ hybridisations were performed. Tissue from infected and healthy roots were

embedded in wax, sectioned, and hybridised to the probes as described by Jackson (1991).

Isolation of 5' termini of mRNAs

The 5' termini of the RNAs of interest were determined by using 5' RACE as described by Frohman et al (1988).

Isolation of promoter regions

The promoter regions of the genes of interest were isolated by vector-ligated PCR. 100ng samples of restriction endonuclease digested C319 genomic DNA were ligated for 4 hours at RT (King and Blakesley, 1986) with 100ng samples of pBluescript (digested with a restriction endonuclease producing compatible termini). Typically enzymes used were *Eco*RI, *Bam*HI, *Hin*dIII, *Bgl*II, *Xho*I, *Cla*I, *Sal*I, *Kpn*I, *Pst*I, *and Sst*I. PCR was then performed on the ligations using a vector primer such as the -40 Sequencing primer and a primer complementary to the 5' terminus of the mRNA. The pCR products were then cloned and sequenced. If necessary the process was repeated with a new primer complementary to the 5' terminus of the promoter fragment to ensure that the control sequences of the promoters were isolated.

Using the procedures described above, a gene, KNT1, was identified and isolated from tobacco plants. A KNT1 promoter fragment of approximately 0.8Kbp in length from the transcription start site, was isolated and inserted into the GUS reporter vector, pBI101 (Jefferson et al, 1987). The

resulting construct, pBIN05101, was used to transform tobacco plants. Upon infection with *M.javanica*, strong GUS expression was observed in the nematode feeding site.

The KNT1 gene was shown to have homologues in species of plant other than tobacco. These include, but are not limited to *Solanum tuberosum, Lycopersicon esculentum* and *Beta vulgaris*. The KNT1 gene is also induced by both root knot and cyst nematode species.

The construct pBIN05101 was deposited by Advanced Technologies (Cambridge) Limited of 210 Cambridge Science Park, Cambridge CB4 0WA, England under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the purposes of Patent Procedure at the National Collections of Industrial, Food and Marine Bacteria (NCIMB), 23 St. Machar Street, Aberdeen, Scotland on 20 March 1997 under accession number NCIMB 40870.

Production of constructs for plant transformation studies, which constructs comprise the maize ribosome inactivating protein and promoters which are induced at and/or adjacent to a nematode feeding site

Constructs comprising the KNT1 nematode-responsive promoter linked to a coding sequence of the maize ribosome inactivating protein and a nos terminator in a pATC derived

plant transformation vector (Figure 10) were used in the transformation studies. These were designed to study the effectiveness of RIP constructs to induce cell death in the feeding sites established by nematodes. The constructs were assembled in the pDVM vector and then the transgene cassettes excised with restriction endonuclease *Not*I and cloned into the binary vector pATC. The constructs were introduced into *Agrobacerium tumefaciens* LBA4404 by electroporation.

The constructs produced were as follows:

1. pATCKNT1/Pro RIP
2. pATCKNT1/RIP-P
3. pATCKNT1/RIP α
4. pATCKNT1/RIP β

A final construct was prepared comprising both the RIP α and the RIP β coding regions under two separate nematode-responsive promoters (Figure 5). The second promoter used was designated KNT2 (see SEQ. ID. No.18). This construct was named:

pATC KNT2/RIP :KNT1/RIP .

Constructs for use in the other embodiments of this invention are similarly prepared with suitable targeting promoters for the alternative appropriate embodiment.

Production of transgenic plants containing maize RIP constructs under the control of nematode-inducible promoters.

All of the constructs above (Figure 5) were introduced into potato cv. Hermes and tobacco cv. K326, by leaf disc co-cultivation using *Agrobacterium tumefaciens* LBA4404 (Horsch et al, 1985).

Transgenic plants of potato and tobacco were generated, and greenhouse nematode resistance trials were conducted.

Resistance screens using Tobacco plants infected with *Meloidogyne javanica*

Transgenic and control tobacco plantlets were planted in a randomised blind experimental design into rootrainers fine potting compost without fertiliser and the larger leaves trimmed by half. The plants were covered with polythene to maintain high humidity whilst weaning. Gradually holes were slit in the polythene to decrease humidity before weaning was completed. One week after weaning, the small plants were infected with 200 hatched J2 *Meloidogyne javanica* nematodes. Watering with liquid feed thereafter was only done once the soil had dried sufficiently to cause the leaves to start

wilting. The rootrainers were placed in trays on heated matting to maintain the soil temperature between 25-30°C. The leaves were trimmed back once a week in order to even out growth and prevent the growing points becoming covered with larger leaves due to density of planting.

The plants were harvested for scoring approximately 8 weeks after infection. Typically, the roots were washed and scored for root growth, level of gall formation and size of galls.

Resistance screens using potato plants infected with *Globodera pallida* pathovar 2/3

Primary resistance trials of transgenic plants were conducted in blind randomised trials on batches of 20 to 25 transgenic lines with at least ten replicates of 2 or more control lines. Rootrainers or containers were filled with a mixture of 50:50 loam and sand. 12 litres of loam and sand is moistened with 1250 mls of water to give a 40% water content. 3 cysts were placed onto the roots of each plantlet which was then inserted into a hole in the compost and the compost gently closed around the roots. The plants were weaned and thereafter, only watered once weekly with liquid feed or when the soil had dried sufficiently. Once the plants reached approx. 10 cm in height, the tips were trimmed off to even out growth.

The plants were grown for approximately three months to permit the cysts to mature. The plants were then allowed to dry out for another month. Cysts were recovered from the plants by washing the soil and roots vigorously in a beaker of 250ml water. The soil was permitted to settle for a few minutes and the supernatant poured into a large filter funnel with a 32cm diameter Whatman No. 1 filter paper disc. The supernatant was left to stand in the funnel for a minute and then the centre of the solution surface was touched with a drop of Hederol detergent to displace the material on the surface meniscus of the solution to the side of the filter. The base of the filter was then pierced to remove the remainder of the solution. The filter disc was removed and the number of cysts adhering to it is counted.

Plants were regarded as showing signs of resistance if they are infected with fewer cysts that susceptible control lines.

Scoring for infection of tobacco and potato with nematodes by staining

Tobacco plants from tissue culture or seedlings were planted and grown as described above with the following modification. Plants were infected with 1000 hatched J2 *Meloidogyne javanica* nematodes. Potato plants were infected with *Globodera pallida* as described above.

One month after infection, cuttings were taken from the plants. The roots were washed clean of soil and bleached for 4 minutes in 1% sodium hypochlorite. The bleach was removed by rinsing with water and then soaking in a large volume of water over 15 minutes with occasional agitation. The roots were then placed in 10 to 15 ml of a 1:500 dilution of acid fuchsin stock solution in 5% acetic acid. (Acid fuchsin stock was prepared according to 'Introduction to Plant Nematology' by V. H. Dropkin, ISBN 0-471-85268-6. Dissolve 0.35g acid fuchsin in 100 mls of 1:3 glacial acetic acid to distilled water). The samples in stain were placed in a boiling water bath for 4 minutes and transferred to 37°C for four hours. The stain was decanted and the samples are cleared by adding acidified glycerol and incubating at 37°C overnight.

The cleared roots with stained nematodes were then mounted in petri-dishes (the sample was placed on the inner side of the lid of a petri dish and the base of the petri dish is used to spread out and compress the sample for easier viewing under the microscope).

The samples were viewed at 20 to 100 x magnification and nematodes were scored in several ways. Root knot nematodes were categorised into three groups: a) vermiform nematodes, b) saccate nematodes that are not producing eggs and c) saccate nematodes producing eggs. The diameters of the essentially saccate nematodes were measured using an eyepiece graticule.

Cyst nematodes were also categorised into three groups: a) vermiform nematodes, b) fat vermiform nematodes that are not producing eggs and c) globose nematodes producing eggs. The diameters of the essentially globose nematodes were measured using an eyepiece graticule.

Resistance effects were measured in terms of absolute numbers of nematodes in root systems, in terms of the proportion of nematodes reaching maturity and producing eggs and in terms of the size of the nematodes.

A number of resistant lines of tobacco and potato were identified expressing maize RIP as a single effector component as well as a two component system

Pot trial of selected Maize RIP potato lines

Chitted tubers of selected potato cv Hermes lines were replicated and used in a standard potato cyst nematode resistance trial with *G.pallida* race 2/3 to which only partial resistance is available. The potatoes comprised four lines transformed with the processed maize RIP-P and four lines transformed with the two component RIPα/RIPβ construct. The trial included untransformed Hermes and Prairie as both stock tubers (control) and material that has been through the same tissue culture procedure as the transgenic plants (control ncc). In addition, the trial included the untransformed control varieties Desiree and Maris Piper (both PCN-

susceptible), and Sante (partial resistance to PCN race 2/3, the best commercially available line).

Table 1 gives a summary of the results expressed as a percentage susceptibility of the transgenic lines relative to non-resistance controls.

There was a large degree of variation in PCN cyst counts both between the controls and between individual replicates, which can be attributed to environmental factors. However, one processed maize RIP line (RIP-P) and one two-component maize RIP line showed a significant reduction in susceptibility relative to the control lines. These are marked by ** in Table 1.

Analysis of nematodes in transgenic tobacco lines expressing maize RIP

Progeny from transgenic tobacco lines transformed with the processed maize RIP-P or the two component maize RIP construct were infected with root knot nematodes. One month after infection, roots from the infected plants were treated with Acid Fuchsin to stain the nematodes *in situ*. Galls and stained nematodes were counted and assigned to the following four categories; empty galls, vermiform juveniles, spherical adults, egg-laying adults (Figure 5).

Whilst there was no significant difference in the total number of nematodes and galls per line between the transgenic

lines and the controls, three transgenic lines, D, G and K, showed fewer egg-laying adults.

In order to determine whether maize RIP lines reduce the growth of nematodes in plants the size of adult nematodes was measured as described above.

Non-egg-laying adult nematodes were significantly smaller for lines D, G & K compared to those on the control plants (Figure 6). However, the sizes of egg-laying nematodes showed much smaller differences (Figure 7), indicating that egg laying appears to occur only when the nematodes have reached a minimum size (of 32 to 36 graticule units). Expression of the RIP appears to slow down the growth of the nematode, resulting in delay in reaching egg-laying size (Figure 8).

In contrast, those lines which did not show any reduction in the number of infecting nematodes or in the number of egg-laying females did not show the decrease in adult female size as observed with lines D, G & K.

These results therefore indicate that the maize RIP and two component maize RIP constructs affect the development of the root knot nematodes.

REFERENCES

Denecke J., Betterman J. and Deblaere R. (1990) The Plant Cell 51-59

Duguid J.R. and Dinauer M.C. (1990) Nucleic Acids Research **18**(9) 2889-2792

Eckert K.A. and Kunkel T.A. (1990) Nucleic Acids Research **18**(13) 3737-3744

Fourney R.M., Miyakoshi J., Day III R.S. and Paterson M.C. (1988) Focus **10**(1) 5-7

Frohman M.A., Dush M.K. and Martin G.R. (1988) Procedings of the Natl. Acad. Sci. USA. **85** 8998-9002

Gawel N.J. and Jarret R.L. (1991) Plant Molec. Biol. Reporter. **9**(3) 262-266

Gussow D. and Clackson T. (1989) Nucleic Acids Research **17** 4000-4008

Horsch R.B., Fry J.E., Hoffman N.L., Eicholtz D., Rogers S.G. and Fraey R.T. (1985) Science **227** 1229-1231

Jackson D. (1991) Molecular Plant Pathology: A Practical Approach. IRL Press, Oxford.

King P.V. and Blakesley R.W. (1986) Focus **8**(1) 1-3

Leborgna-Castel N., Jelitto-Van Droven E.P.W.M., Crofts A.J. and Denecke J. (1999) The Plant Cell 11 459-469

Maniatis T., Fritsch E.F. and Sambrook J. (1982) Molecular Cloning; A Laboratory Manual. N.Y. Cold Spring Harbour Laboratory.

Mariani et al (1990) Nature **347** 737-741

O'Reilly D., Thomas C.J.R. and Coutts R.H.A. (1991) J. Gen. Virology **17** 1-7

Perry S.E., Nichols K.W. and Fernandez D.E. (1996) The Plant Cell **8** 1977-1989

Samach A., Kohlami S.E., Motte P., Datla R. and Haughn G.W. (1997) The Plant Cell **9** 559-570

Shirzadegan M., Christie P. and Seemann J.R. (1991) Nucleic Acids Research **19** (21) 6055

Sieburth L.E. and Meyerowitz E.M. (1997) The Plant Cell **9** 355-365

Stirpe and Barbieri (1986) FEBS Lett. **195**, 1-8.

Stirpe *et al* (1978)FEBS Lett. **85** 65-67.

Twell et al (1991) Molec. Gen. Genet. **217** 240-245

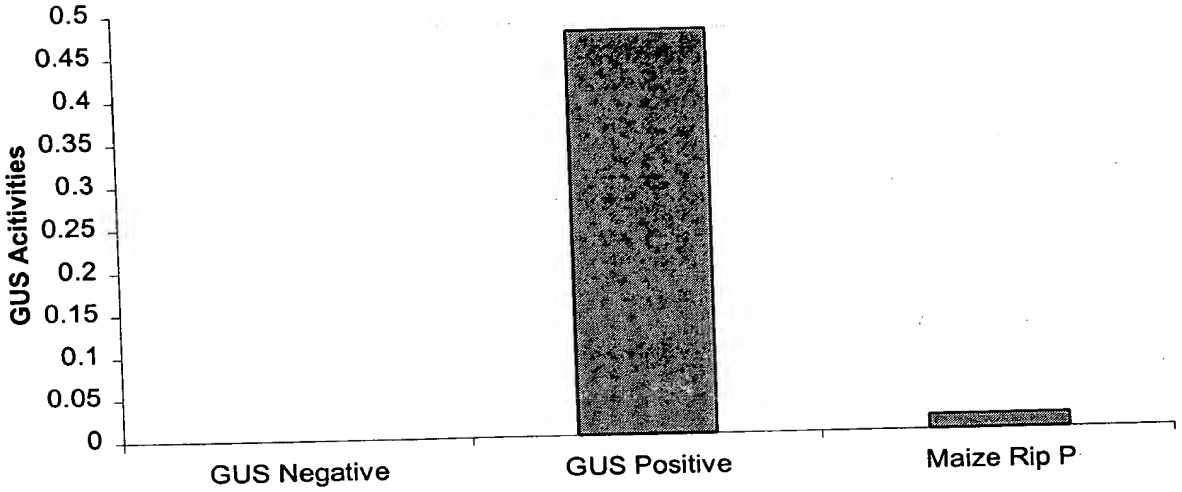


Figure 1. Effect of maize RIP P protein on tobacco ribosomes as measured by the GUS protein synthesis

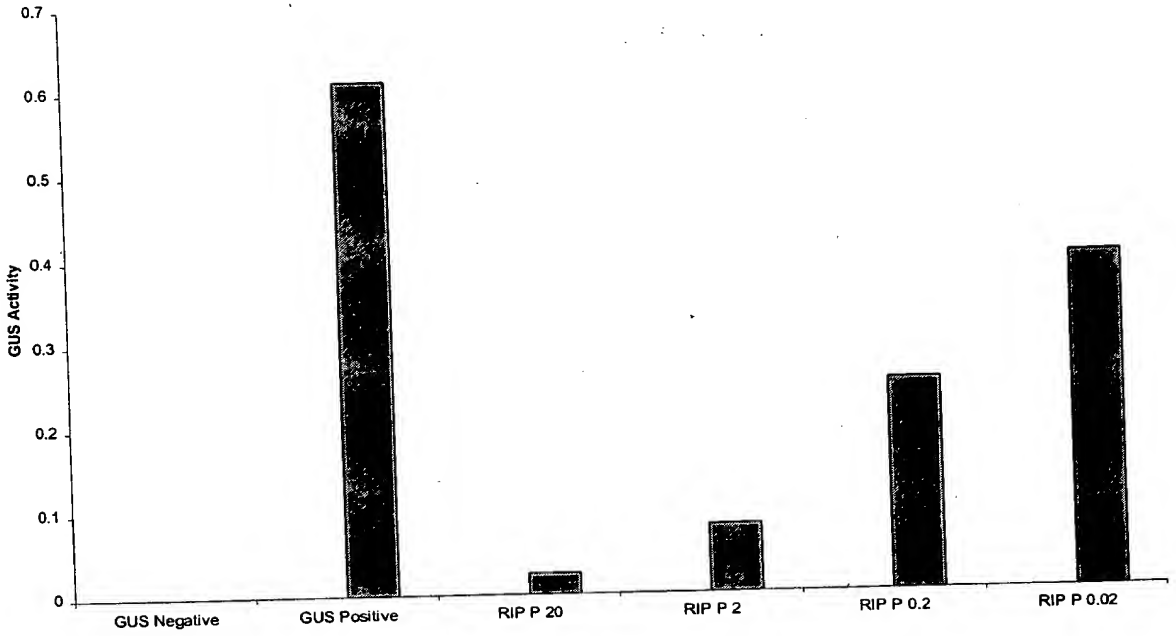


Figure 2. Effect of different amounts of maize RIP DNA (20µg to 0.02µg) on tobacco ribosomes as measured by the GUS protein synthesis

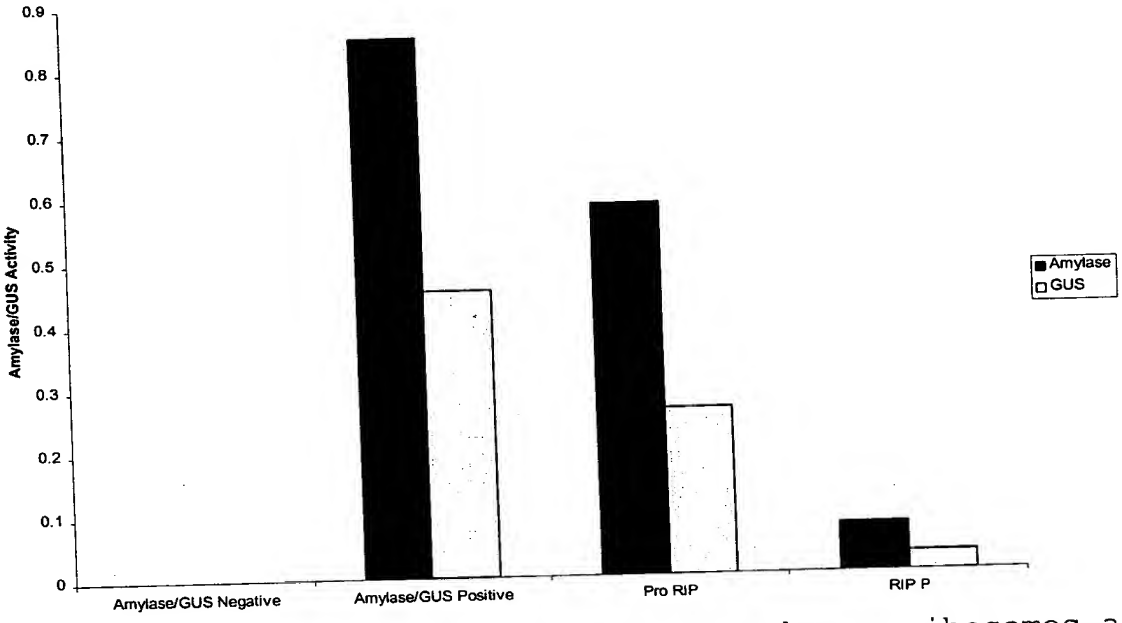


Figure 3. Effect of maize RIP proteins on tobacco ribosomes as measured by the αAmylase and GUS protein synthesis

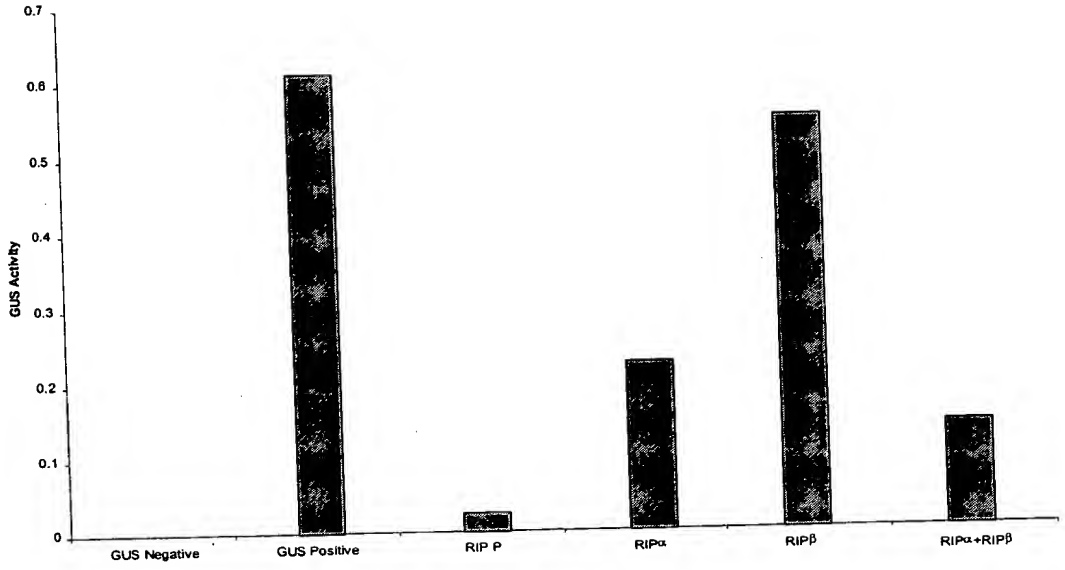


Figure 4. Effect of maize RIP α and β polypeptides regions on tobacco ribosomes as measured by the GUS protein synthesis

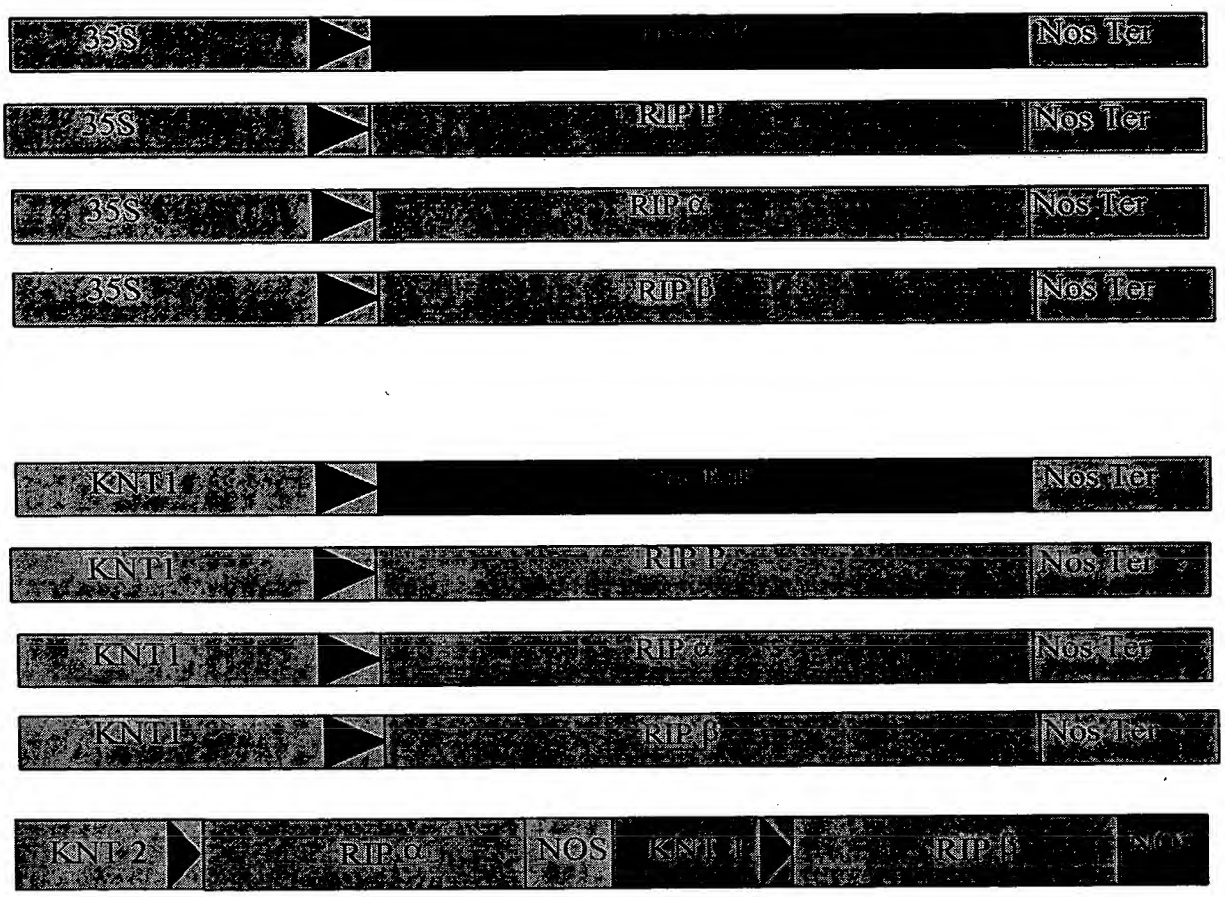


Figure 5. Schematic Diagram of the pDVM35S and pATCKNT1 promoter constructs for expression of maize RIPs

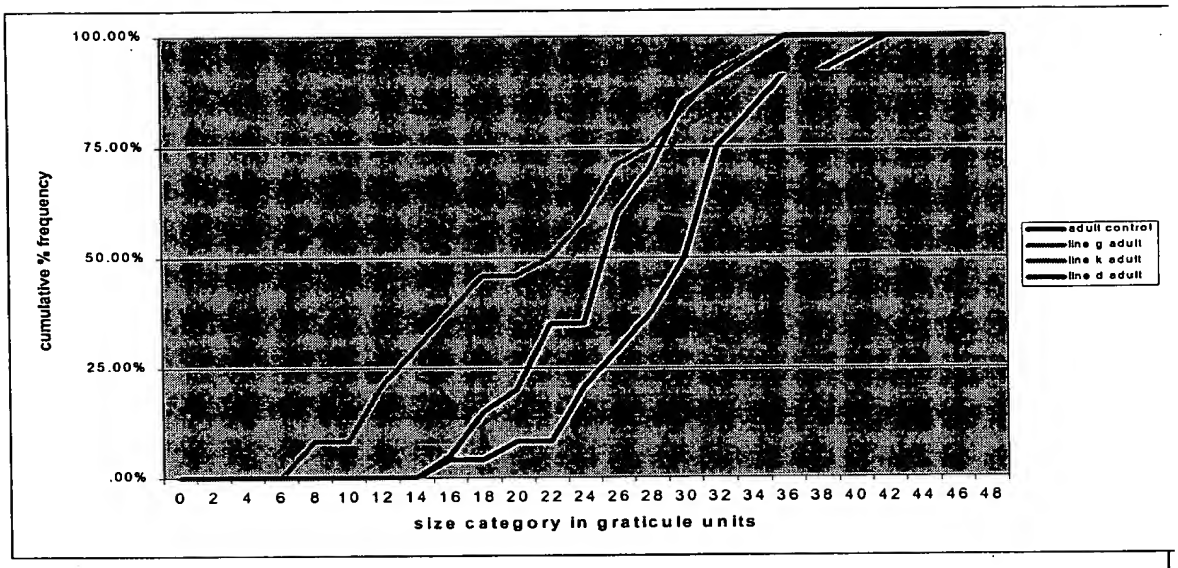


Figure 6. Adult nematode sizes in control and transgenic maize RIP lines plotted as a cumulative frequency graph.

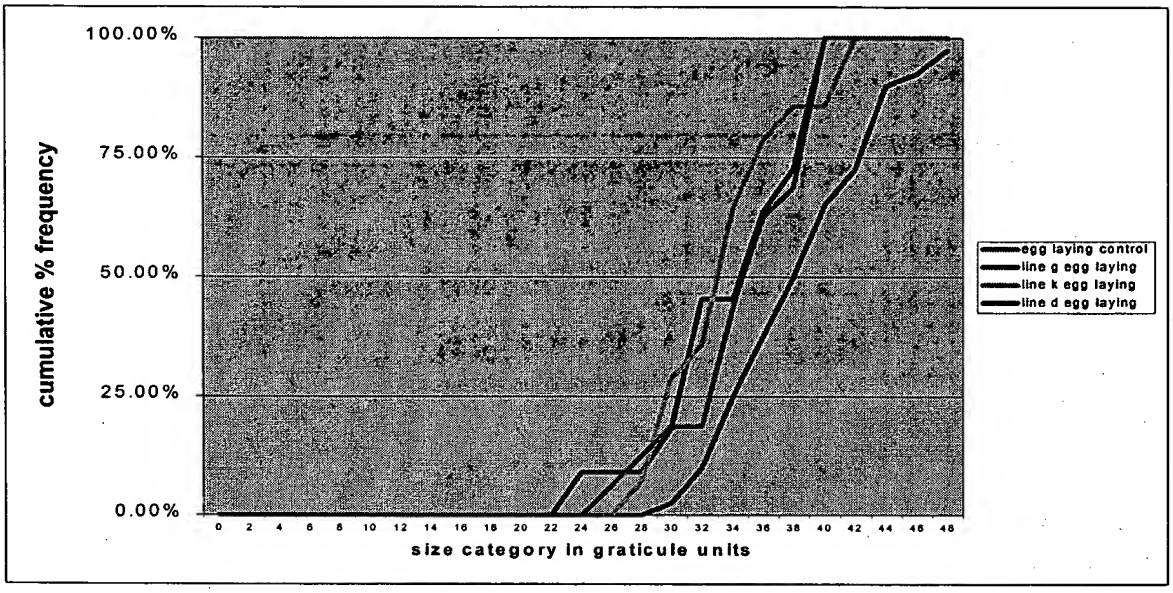


Figure 7 Egg laying female sizes in control and transgenic maize RIP lines plotted as a cumulative frequency graph

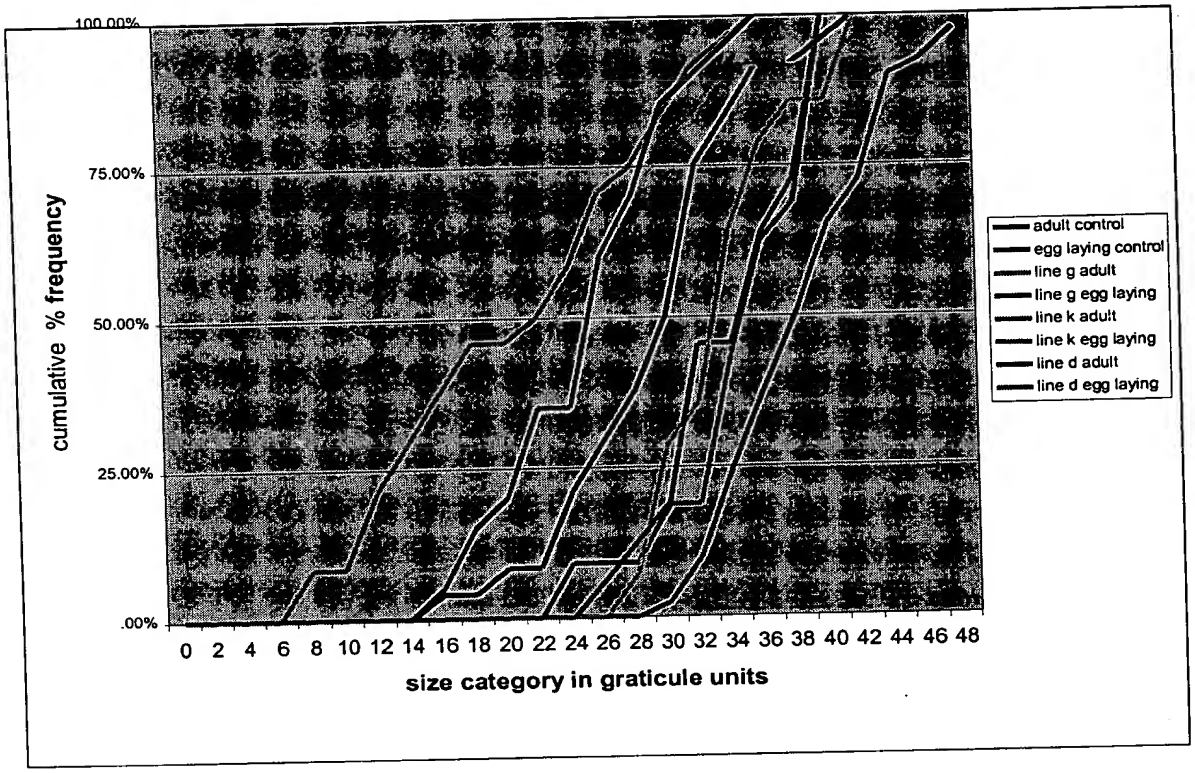


Figure 8. Comparison between the sizes of adult and egg-laying root knot nematodes in control and transgenic lines.

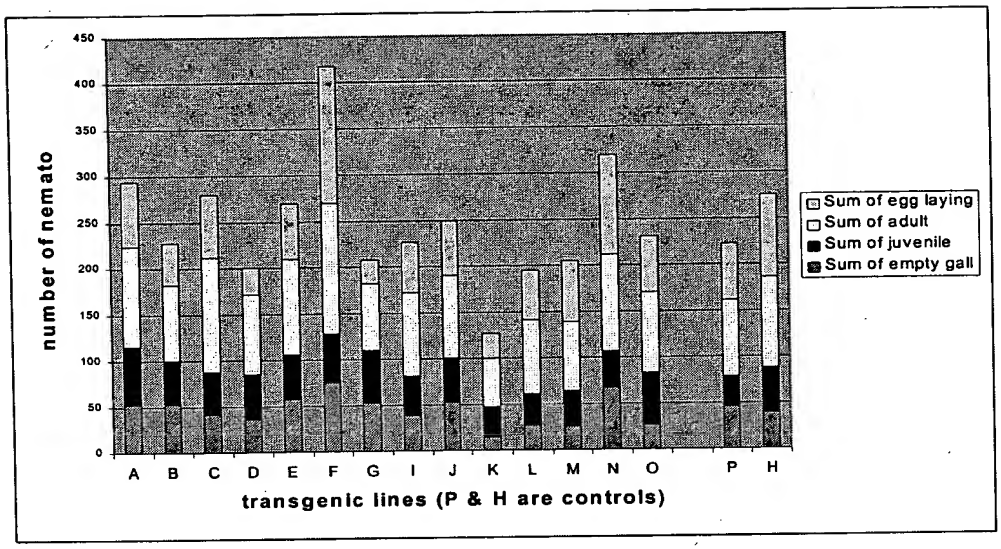


Figure 9. Number of galls and nematodes in three categories for each transgenic tobacco line. Lines A to G are progeny from maize RIP-P transformants, lines I to Q are progeny from two component maize RIPα / RIPβ transformants. Lines P & H are untransformed control lines.

Figure 10. Plant transformation vector pATC

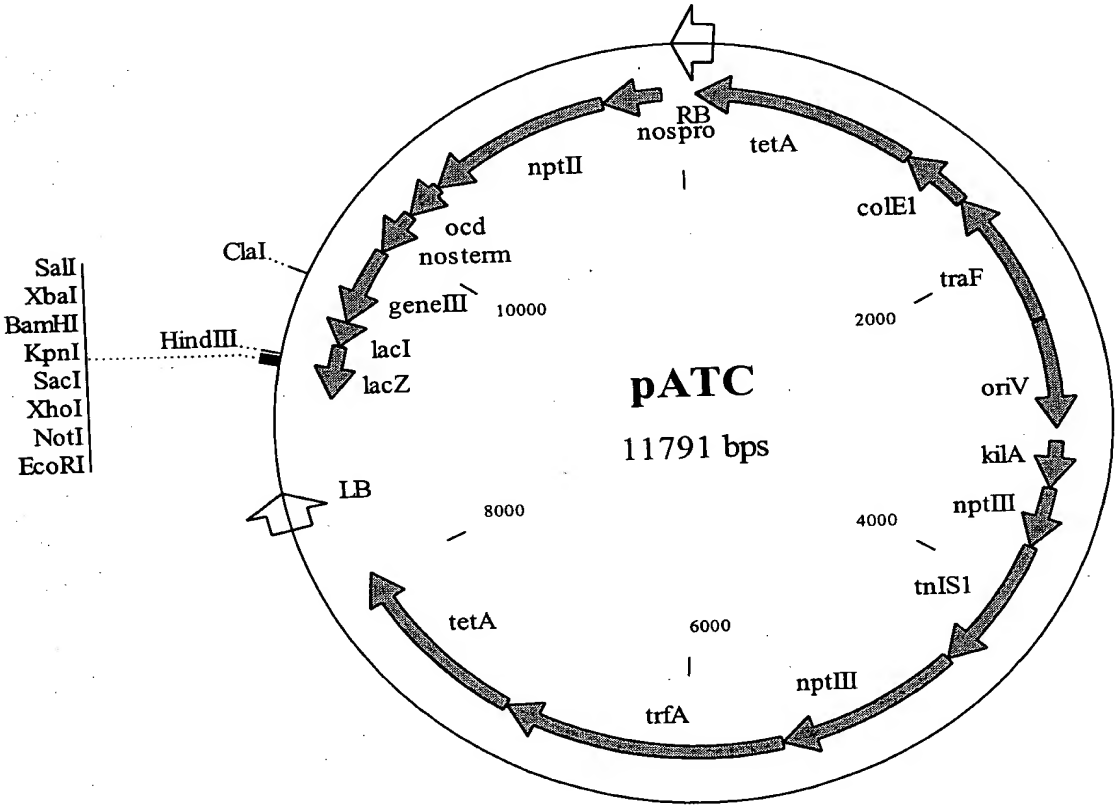


Figure 11. Cloning vector pDVM.

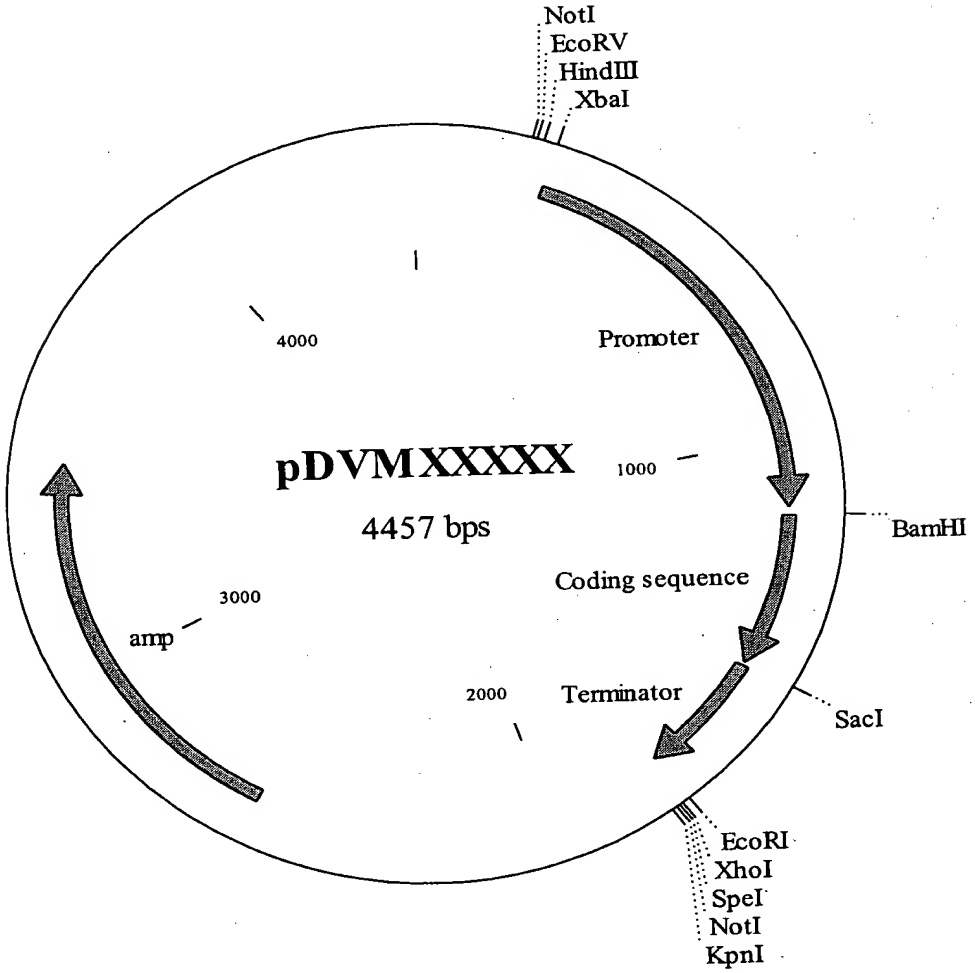
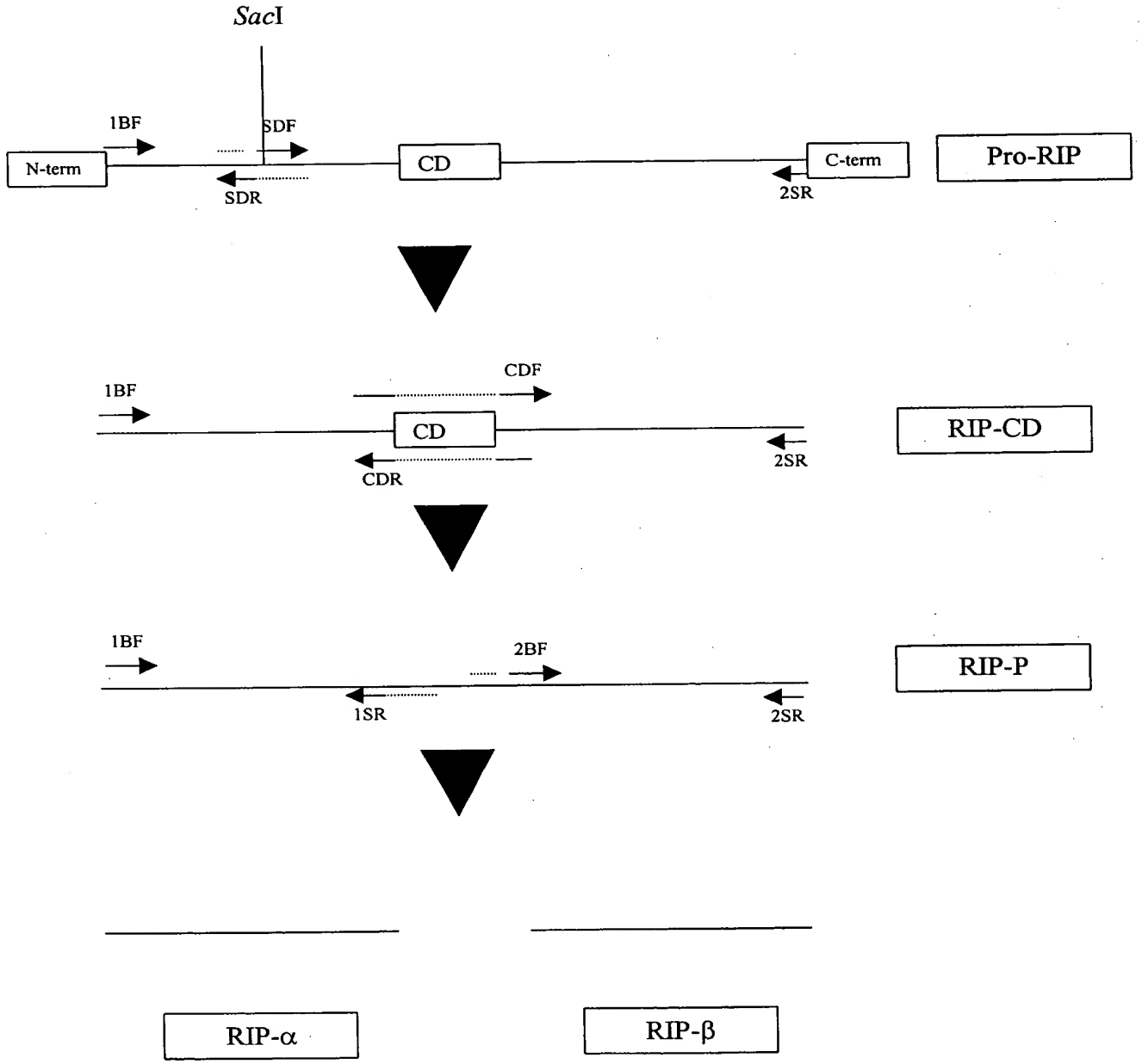


Figure 12. Schematic diagram of the production of maize RIP variants by PCR



Construct	Cultivar	Line	Percentage Susceptibility
RIP P	Hermes	1	143
RIP P	Hermes	10	72
RIP P	Hermes	13	45**
RIP P	Hermes	15	63
RIP α / RIP β	Hermes	9	44**
RIP α / RIP β	Hermes	11	65
RIP α / RIP β	Hermes	14	103
RIP α / RIP β	Hermes	15	88
NCC Control	Hermes		90
Control	Hermes		139
NCC Control	Prairie		115
Control	Prairie		93
Control	Desiree		94
Control	Maris Piper		62
Control	Sante		21

Table 1. Results of PCN trial. Percentage susceptibility relative to mean of all non-resistant controls. Lines showing reduced susceptibility marked **.

SEQ. ID. No. 1

Maize Pro-RIP Nucleotide sequence:

atg**GCCGAGA TAACCCTAGA GCCGAGTGAT CTTATGGCGC AAACAAAC**AA
AAGAATAGTG CCAAAGTTCA CTGAAATCTT CCCCGTGGAG GACGCGAACT
ACCCTTACAG CGCCTTCATC GCGTCGGTCC GGAAAGACGT GATCAAACAC
TGCACCGACC ATAAAGGGAT CTTCCAGCCC GTGCTGCCAC CGGAGAAGAA
GGTCCCGGAG CTATGGTTCT ACACA*GAACT G*AAAACTAGG ACCAGCTCCA
TCACGCTCGC CATACGCATG GACAACCTGT ACCTCGTGGG CTTCAGGACC
CCGGGCGGGG TGTGGTGGGA GTTCGGCAAG GACGGCGACA CCCACCTCCT
CGGCGACAAC CCCAGGTGGC TCGGCTTCGG CGGCAGGTAC CAGGACCTCA
TCGGCAACAA GGGTCTGGAG ACCGTCACCA TGGGCCGCGC CGAAATGACC
AGGGCCGTCA ACGACCTGGC GAAGAAGAAG AAG**ATGGCGA CACTGGAGGA**
GGAGGAGGTG AAGATGCAGA TGCAGATGCC GGAGGCCGCT GATCTGGCGG
CGGCGGCAGC GGCTGACCCA CAGGCCGACA CGAAGAGCAA GCTGGTGAAG
CTGGTGGTCA TGGTGTGCGA GGGGCTGCGG TTCAACACCG TGTCCCGCAC
GGTGGACGCG GGGTTCAACA GCCAGCACGG GGTGACCTTG ACCGTGACGC
AGGGGAAGCA GGTGCAGAAG TGGGACAGGA TCTCCAAGGC GGCCTTCGAG
TGGGCTGACC ACCCCACCGC TGTGATCCCC GACATGCAGA AGCTTGGCAT
CAAGGATAAG AACGAAGCAG CGAGGATCGT TGCGCTCGTT AAGAATCAAA
CTACTGCCGC TGCC**GCTACT GCTGCCAGTG CTGACAACGA CGACGACGAG**
GCCtaataa

The N-terminal, Central, and C-terminal domains are indicated by bold text.

The sequence replacing the removed *Sac*I site is indicated by italicised text.

The Methionine and stop codons added via the PCR primers are indicated in lower case text.

SEQ. ID. No. 2

Maize RIP-P nucleotide sequence:

atgAAAAGAA TAGTGCCAAA GTTCACTGAA ATCTTCCCCG TGGAGGACGC

GAACTACCCT TACAGCGCCT TCATCGCGTC GGTCCGGAAA GACGTGATCA

AACACTGCAC CGACCATAAA GGGATCTTCC AGCCCGTGCT GCCACCGGAG

AAGAAGGTCC CGGAGCTATG GTTCTACACA *GAACTG*AAAA CTAGGACCAG

CTCCATCACG CTCGCCATAC GCATGGACAA CCTGTACCTC GTGGGCTTCA

GGACCCCGGG CGGGGTGTGG TGGGAGTTCG GCAAGGACGG CGACACCCAC

CTCCTCGGCG ACAACCCCAG GTGGCTCGGC TTCGGCGGCA GGTACCAGGA

CCTCATCGGC AACAAGGGTC TGGAGACCGT CACCATGGGC CGCGCCGAAA

TGACCAGGGC CGTCAACGAC CTGGCGAAGA AGAAGAAGGC GGCTGACCCA

CAGGCCGACA CGAAGAGCAA GCTGGTGAAG CTGGTGGTCA TGGTGTGCGA

GGGGCTGCGG TTCAACACCG TGTCCCGCAC GGTGGACGCG GGGTTCAACA

GCCAGCACGG GGTGACCTTG ACCGTGACGC AGGGGAAGCA GGTGCAGAAG

TGGGACAGGA TCTCCAAGGC GGCCTTCGAG TGGGCTGACC ACCCCACCGC

TGTGATCCCC GACATGCAGA AGCTTGGCAT CAAGGATAAG AACGAAGCAG

CGAGGATCGT TGCGCTCGTT AAGAATCAAA CTACTGCCGC TGCCtaataa

The sequence replacing the removed *Sac*I site is indicated by italicised text.

The Methionine and stop codons added via the PCR primers are indicated in lower case text.

SEQ. ID. No. 3.

Maize RIP α nucleotide sequence:

atgAAAAGAA TAGTGCCAAA GTTCACTGAA ATCTTCCCCG TGGAGGACGC

GAACTACCCT TACAGCGCCT TCATCGCGTC GGTCCGGAAA GACGTGATCA

AACACTGCAC CGACCATAAA GGGATCTTCC AGCCCGTGCT GCCACCGGAG

AAGAAGGTCC CGGAGCTATG GTTCTACACA ***GAACTG***AAAA CTAGGACCAG

CTCCATCACG CTCGCCATAC GCATGGACAA CCTGTACCTC GTGGGCTTCA

GGACCCCGGG CGGGGTGTGG TGGGAGTTCG GCAAGGACGG CGACACCCAC

CTCCTCGGCG ACAACCCCAG GTGGCTCGGC TTCGGCGGCA GGTACCAGGA

CCTCATCGGC AACAAGGGTC TGGAGACCGT CACCATGGGC CGCGCCGAAA

TGACCAGGGC CGTCAACGAC CTGGCGAAGA AGAAGAAGta ataa

The sequence replacing the removed *Sac*I site is indicated by italicised text.

The Methionine and stop codons added via the PCR primers are indicated in lower case text.

SEQ. ID. No. 4

Maize RIP β nucleotide sequence:

atgGCGGCTG ACCCACAGGC CGACACGAAG AGCAAGCTGG TGAAGCTGGT

GGTCATGGTG TGCGAGGGGC TGCGGTTCAA CACCGTGTCC CGCACGGTGG

ACGCGGGGTT CAACAGCCAG CACGGGGTGA CCTTGACCGT GACGCAGGGG

AAGCAGGTGC AGAAGTGGGA CAGGATCTCC AAGGCGGCCT TCGAGTGGGC

TGACCACCCC ACCGCTGTGA TCCCCGACAT GCAGAAGCTT GGCATCAAGG

ATAAGAACGA AGCAGCGAGG ATCGTTGCGC TCGTTAAGAA TCAAACTACT

GCCGCTGCCG CTACTGCTGC CAGTGCTGAC AACGACGACG ACGAGGCCta

ataa

The Methionine and stop codons added via the PCR primers are indicated in lower case text.

Maize RIP Primers

(Lower case type indicated introduced restriction sites)

SEQ. ID. No.5.

PRORIPBF:

actcgagtctagaggatcc**ATGGCCGAGATAACCCTAGAGCCG**

SEQ. ID. No. 6

PRORIPSR:

gactagtgtcgacgagctc**TTATTAGGCCTCGTCGTCGTCGTTGTCAGC**

SEQ.ID. No. 7

RIP1BF:

gctcgagtctagaggatccATGAAAAGAATAGTGCCAAAGTTCACTG

SEQ. ID. No. 8

RIP2SR:

gactagtgtcgacgagctcTTATTAGGCAGCGGCAGTAGTTTGATTCTTAACG

SEQ. ID. No. 9

RIP1SR:

aactagtgtcgacgagctcTTATTACTTCTTCTTCTTCGCCAGGTCGTTGACG

SEQ. ID. No. 10

RIP2BF:

actcgagtctagaggatccATGGCGGCTGACCCACAGGCCGACACGAAGAG

Overlapping primers for the removal of central domain (CD) region:

SEQ. ID. No. 11

RIPCDF:

GACCTGGCGAAGAAGAAGAAGGCGGCTGACCCACAGGCCGAC

SEQ. ID. No. 12

RIPCDR:

GTCGGCCTGTGGGTCAGCCGCCTTCTTCTTCTTCGCCAGGTC

Sac I modification primers:

Upper case type indicates nucleotides modified to remove the *Sac*I site

SEQ. ID. No. 13

RIPSDF:

ccggagctatggttctacaca**GAACTG**aaaactaggaccagctcc

SEQ. ID. No. 14

RIPSDR:

ggagctggtcctagttttt**CAGTTC**tgtgtagaaccatagctccgg

cDNA cloning oligonucleotides

SEQ. ID. No. 15

SUB21

CTCTTGCTTGAATTCGGACTA

SEQ. ID. No. 16

SUB25

TAGTCCGAATTCAAGCAAGAGCACA

SEQ. ID. No. 17

LDT15

GACAGAAGCGGATCCTTTTTTTTTTTTTTT

SEQ. ID. No. 18

The KNT2 Promoter nucleotide sequence

TCTAGAAAGC TTATCTAAAC AAAGTTTTAA ATTCATTTCT TAAACGTCCA

TTACAATGTA ATATAACTTA GTCGTCTCAA TTAAACCATT AATGTGAAAT

ATAAATCAAA AAAAGCCAAA GGGCGGTGGG ACGGCGCCAA TCATTTGTCC

TAGTCCACTC AAATAAGGCC CATGGTCGGC AAAACCAAAC ACAAAATGTG

TTATTTTTAA TTTTTTCCTC TTTTATTGTT AAAGTTGCAA AATGTGTTAT

TTTTGGTAAG ACCCTATGGA TATATAAAGA CAGGTTATGT GAAACTTGGA

AAACCATCAA GTTTTAAGCA AAACCCTCTT AAGAACTTAA ATTGAGCTTC

TTTTGGGGCA TTTTTCTAGT GAGAAGGATC C

Appendix

Maize Pro RIP

PRORIPBF

MAEITLEPSDLMAQTNKRIVPKFTEIFPVEDANYPYSAFIASVRKDVIK

RIPSDF and RIPSDR

HCTDHKGIFQPVLPPEKKVPELWFYTELKTRTSSITLAIRMDNLYLVGFRTPGGVWWEFGKD

GDTHLLGDNPRWLGFGGRYQDLIGNKGLETVTMGRAEMTRAVNDLAKKKKMATLEEEEVKMQ

MQMPEAADLAAAAAADPQADTKSKLVKLVVMVCEGLRFNTVSRTVDAGFNSQHGVTLTVTQG

KQVQKWDRISKAAFEWADHPTAVIPDMQKLGIKDKNEAARIVALVKNQTTAAAATAASADND

DDEA**

PRORIPSR

Blue: N terminal, C terminal and Central domain (CD) processed segments

** Two stop codons: TAA and TAA were added through primers

Boxed Regions: Primers for PCR amplification

EL: Sac I restriction site

Pro RIP gene was amplified from Maize genomic DNA isolated from seedling leaves using PCR primers with suitable restriction sites.

To remove the Sac I restriction site in the gene, Pro RIP was first amplified from genomic DNA in two parts with mutagenic Sac I PCR primers (RIPSDF and RIPSDR) along with PRORIPBF and PRORIPSR primers. Full length Pro RIP was then PCR amplified from the above two PCR fragments by Splicing by Overlap Extension (SOE) technique with PRORIPBF and PRORIPSR primers and cloned into different vectors.

Maize RIP Delta N and C (RIP CD)

RIP1BF

MKRIVPKFTEIFPVEDANYPYSAFIASVRKDVIKHCTDHKGIFQPVLPP

RIPSDF and RIPSDR

EKKVPELWFYTELKTRTSSITLAIRMDNLYLVGFRTPGGVWWEFGKDGDTHLLGDNPRWLGF

GGRYQDLIGNKGLETVTMGRAEMTRAVNDLAKKKKMATLEEEEVKMQMQMPEAADLAAAAAA

DPQADTKSKLVKLVVMVCEGLRFNTVSRTVDAGFNSQHGVTLTVTQGKQVQKWDRISKAAFE

WADHPTAVIPDMQKLGIKDKNEAARIVALVKNQTTAA**

RIP2SR

Blue: Central domain (CD) processed segment

M and ** Methionine and Stop codons: TAA and TAA were added through primers

Boxed Regions: Primers for PCR amplification

EL: Sac I restriction site

Maize RIP CD gene was amplified from Maize genomic DNA isolated from seedling leaves using PCR primers with suitable restriction sites.

To remove the Sac I restriction site in the gene, RIP CD was first amplified from genomic DNA in two parts with mutagenic Sac I PCR primers (RIPSDF and RIPSDR) along with RIP1BF and RIP2SR primers. Full length RIP CD was then PCR amplified from the above two PCR fragments by Splicing by Overlap Extension (SOE) technique with RIP1BF and RIP2SR primers and cloned into different vectors.

Maize RIP (RIP P)

RIP1BF

MKRIVPKFTEIFPVEDANYPYSAFIASVRKDVIKHCTDHKGIFQPVLPPEKKVPELWFYTEL

KTRTSSITLAIRMDNLYLVGFRTPGGVWWEFGKDGD

RIPCDF

THLLGDNPRWLGFGGRYQDLIGNKGLETVTMGRAEMTRAVNDLAKKKKM

RIPCDR

ATLEEEEVKMQMQMPEAADLAAAAAADPQADTKSKLVKLVVMVCEGLRFNTVSRTVDAGFNS

QHGVTLTVTQGKQVQKWDRISKAAFEWADHPTAVIPDMQKLGIKDKNEAARIVALVKNQTTA

A**

RIP2SR

M and **: Methionines and Stop codons (TAA) were added through primers

Blue: Central domain

Boxed Regions: Primers for PCR amplification

Processed maize RIP (RIP P) was amplified from partially processed RIP (RIP CD) clone plasmid DNA.

To remove the Central domain region (CD) from processed RIP version (RIP P), RIP P was first amplified from RIP CD clone plasmid DNA, in two parts, with overlapping primers designed around the central domain region (RIPCDF and RIPCDR) along with RIP1BF and RIP2SR primers. Full length RIP P was then PCR amplified from the above two PCR fragments by Splicing by Overlap Extension (SOE) technique with RIP1BF and RIP2SR primers and cloned into different vectors.

Maize RIP α and RIP β

RIP1BF

MKRIVPKFTEIFPVEDANYPYSAFIASVRKDVIKHCTDHKGIFQPVLPPEKKVPELWFYTEL

KTRTSSITLAIRMDNLYLVGFRTPGGVWWEFGKDGD

RIP1SR

THLLGDNPRWLGFGGRYQDLIGNKGLETVTMGRAEMTRAVNDLAKKKK**

RIP2BF

MAADPQADTKSKLVKLVVMVCEGLRFNTVSRTVDAGFNSQHGVTLTVTQGKQVQKWDRISKA

AFEWADHPTAVIPDMQKLGIKDKNEAARIVALVKNQTTAAA**

RIP2SR

M and **: Methionines and Stop codons (TAA) were added through primers

Green: RIP α Red: RIP β

Boxed Regions: Primers for PCR amplification

RIP α and RIP β polypeptide domains were amplified from RIP P clone plasmid DNA using RIP1BF+RIP1SR and RIP2BF+RIP2SR primer pairs and cloned into different vectors.